

A Study of Enzymes that can Break Down Tobacco-Leaf Components

4. MAMMALIAN PANCREATIC AND SALIVARY ENZYMES

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In other papers of this series (Holden, Pirie & Tracey, 1950; Holden, 1950*a*, *b*) the effects of a number of polysaccharases on leaf fibre are described. The only polysaccharase recognized in tobacco leaves in significant quantity is amylase (Frankenburg, 1946), and even this is not present in sufficient quantity to effect complete removal of starch on repeated prolonged incubation. It is convenient to remove the leaf starch so that varying amounts of starch hydrolysis by the leaf amylase will not complicate the results observed with other enzymes. This has been done with salivary amylase. Some of the enzyme preparations studied do not contain protease in significant amounts. Trypsin has accordingly been used in the form of a crude preparation from pancreas in order to remove protein. This 'trypsin' contains amylase, and the effect of the other enzymes present in it may be made clearer by preliminary treatment of the substrate with salivary amylase.

The protease in tobacco-leaf fibre has already been described (Tracey, 1948). The low activities found will be still lower under the conditions used in the present work, for no reducing agents were added and experiments were carried out at pH 7, whereas the pH optimum of the leaf protease is about 5. The effect of tobacco protease can therefore be ignored. Commercial trypsin softens the fibre. This softening is probably due to the protease component of the mixture, for similar effects are obtained by the use of crystalline chymotrypsin (Bawden & Pirie, 1946).

MATERIALS AND METHODS

The preparation of the tobacco-leaf fibre, preparation of fractions for analysis and analytical methods have been described by Holden *et al.* (1950).

Enzyme solutions. Human saliva was collected, centrifuged and the clear supernatant liquid was stored at 5° with the addition of CHCl_3 . Total carbohydrate contents were 0.25–0.40 g./l., and dry matter 6–9 g./l. Trypsin solutions were made up from dry pancreas preparations (British Drug Houses Ltd. or Hopkin & Williams) by suspending 1 g. in 100 ml. water and centrifuging after standing for 1 hr. These solutions contained 7–8 g. dry matter/l. and 0.8–0.9 g. total N/l.

Incubations with enzyme were carried out at 35° in the presence of CHCl_3 . Details of volume of solution, buffer, pH, and time are given with each set of experiments.

EXPERIMENTS AND RESULTS

LEAF STARCH

The starch content of the fibre used lay between 5 and 30% of the dry weight. It varies with illumination at the time of harvesting, and also depends to some extent on the efficiency of the grinding of the leaves, for starch appears in the sap fraction. The more thorough the mincing, the larger is the proportion of starch lost from the fibre. Much of the starch in tobacco leaves is in approximately circular grains 2–10 μ . in diameter, and about 1 μ . thick. In polarized light the grains show weak birefringence when examined in water. With I_2 in KI solution the grains give the usual deep-purple colour.

Tobacco-leaf amylase

Hydrolysis of leaf starch by amylase present in the fibre occurs on incubation at pH 7. This action is not complete; even after repeated incubation the total amount of carbohydrate liberated is less than the total got with added amylase (Table 1). In addition to amylase, maltase appears to be present, for the reducing sugar calculated as glucose found in solution usually accounts for about 90% of the total carbohydrate. Soluble starch and maltose are also hydrolysed by a suspension of leaf fibre. Leaf amylase is present in the chloroplast fraction of the sap and will be discussed in the section dealing with the action of trypsin on this fraction.

Salivary amylase

The action of salivary amylase was measured by incubating fibre at pH 7 with 1 ml. saliva/g. wet wt. of fibre. Acetate-veronal-NaCl buffer (Michaelis, 1931), or phosphate buffer containing added NaCl was used to maintain pH. Salivary amylase will attack isolated tobacco-leaf starch that has not been boiled or ground, but after boiling the starch is attacked much more rapidly. Boiled leaf starch (50 mg.) with 1 ml. saliva gave no colour with I_2 after 10 min. at room temperature, whereas unboiled starch under the same conditions took 6 hr. to reach the achromic point. After incubation overnight, unboiled potato starch was almost unattacked by saliva.

Starch in unboiled fibre is attacked by salivary amylase (Table 1), and after three incubations of 24 hr. at pH 7 at 35° (renewing the saliva for each incubation), the amount of carbohydrate liberated by further treatments with saliva is negligible. When the tobacco fibre is boiled before the action of salivary amylase is begun, the starch is digested more rapidly than in unboiled fibre, in spite of the fact that in unboiled fibre the leaf amylase is still active. The total amount of carbohydrate liberated in three incubations is not affected by boiling. Soluble carbohydrate is also liberated

Table 1. *Liberation of soluble carbohydrate from fresh and boiled tobacco-leaf fibre with and without added salivary amylase*

(2 g. lots of fibre (dry matter 23.2%) + 5 ml. *m*-NaCl + 8 ml. 0.2*M*-sodium phosphate buffer (pH 7) + 5 ml. water + 2 ml. saliva. Saliva was replaced by water in the controls. Separate lots for the incubations up to 16.5 hr. Other lots incubated three times for 24 hr. each.)

				mg. carbohydrate liberated/100 mg. dry fibre			
				Fresh		Boiled	
				With saliva	Control	With saliva	Control
Boiled fibre extract		—	—	6.9	8.4
0 hr.				—	0.60	—	Approx. 0.1
1 hr.				1.38	—	7.03	—
2 hr.				2.60	—	8.06	—
4 hr.				3.32	—	8.50	—
16.5 hr.				8.13	0.91	10.30	1.91
First incubation (24 hr.)				9.85	0.86	12.10	3.75
Second incubation (24 hr.)				3.52	0.33	2.44	3.44
Third incubation (24 hr.)				1.85	0.12	1.45	2.86
Totals for three 24 hr. incubations				15.22	1.31	15.99	10.05

from boiled fibre on incubation without added amylase (Table 1). The reducing sugar content is then small compared with the total soluble carbohydrate, and the solution gives a purple colour with I_2 . The material found in solution appears to be starch rendered soluble by boiling and leached out during incubation.

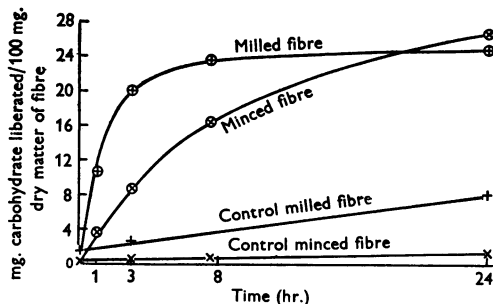


Fig. 1. Liberation of carbohydrate from milled and minced tobacco-leaf fibre on incubation with saliva. 1 g. lots of fibre (dry matter 28.6%) + 4 ml. 0.2*M*-sodium phosphate buffer (pH 7) + 2 ml. *m*-NaCl + 1 ml. water + 1 ml. saliva. Controls without saliva. Incubated for different times.

Effect of milling. Increasing the subdivision of the fibre by grinding with the triple-roller mill increases accessibility of the starch both to salivary amylase, and to the leaf amylase. This is shown in Fig. 1. Milling releases some carbohydrate from the fibre, and it is clear that on subsequent incubation the release is more rapid with milled than with minced fibre. The same is true with added salivary amylase, although after long incubation both reach approximately the same end point. There is thus no evidence that any substrate for these enzymes exists in the minced leaf in a position where it is inaccessible. The effect of milling is discussed further in the section on pancreatic protease.

Effect of microtoming. Fibre was also subdivided by embedding in paraffin wax, and cutting the block of wax on a microtome into sections about 5 μ . thick. The fibre was

recovered from the sections by solution of the wax in light petroleum. Samples of the sectioned fibre were treated with both saliva and trypsin, and the amount of carbohydrate liberated was not significantly different from that liberated from whole fibre. However, any advantage got by increased regular subdivision of the fibre may have been obscured by the possibly deleterious effect of the heat treatment necessary in its preparation.

Fat extraction. Extraction of fibre with neutral or acid ethanol-ether mixtures before incubation with saliva had no effect on the rate of release of soluble carbohydrate, indicating the absence of a fat-soluble amylase inhibitor coating the grains, such as is found in some seeds.

Table 2. *Effect of shaking on liberation of carbohydrate from tobacco-leaf fibre by salivary amylase*

(500 mg. lots of fibre (dry matter 24.8%) + 2 ml. 0.2*M*-sodium phosphate buffer (pH 7) + 1 ml. *m*-NaCl + 0.5 ml. water + 0.5 ml. saliva. Saliva replaced by water in the controls. Incubated for different times at 35°. One series shaken in tubes which were inverted four times a minute throughout the incubation period, one series not shaken.)

mg. carbohydrate liberated/100 mg. dry fibre				
Incubation time (hr.)	Not shaken		Shaken	
	With saliva	Control	With saliva	Control
0	—	1.10	—	1.10
1	1.26	—	2.26	—
2	1.81	—	4.39	—
4	4.68	—	7.07	—
7.5	7.45	—	9.25	—
23.25	9.72	1.52	9.55	1.58

Shaking during enzyme action. Carbohydrate is liberated more rapidly when the contents of the tube are shaken, but the same total amount is liberated when incubations are continued long enough (Table 2).

Pancreatic amylase

Commercial trypsin solution (1 ml.) was used with 1 g. wet wt. of fibre, the other conditions being those used in the salivary amylase experiments. The trypsin solutions were not dialysed because this resulted in a loss of amylase activity. The amylase activity of the nominally 1% (w/v) solution used was found to be about equivalent to that of undiluted saliva, as measured by its action on soluble starch and on fresh fibre. Its action on boiled and milled fibre was similar to that of salivary amylase. Either enzyme used on minced fibre after the exhaustive action of the other liberated no more carbohydrate.

chloroplasts, since these seem to represent its main component, contains also minute particles that are strongly birefringent; these are calcium oxalate. The approximate composition of the fraction is given in Table 4. Since this fraction appears to be similar to one of the three present in fibre, the ratio of the amount of green pigment extractable with acetone to the insoluble N in sap sediment and in fibre was determined. Sap was centrifuged for 10 min. at 1500 g immediately after the leaves had been minced. The supernatant, which was rejected, was still coloured green by finely divided chromoprotein, which flocculates together with some soluble sap proteins on ageing.

The sediment was resuspended in distilled water and

Table 3. *Liberation of nitrogen and carbohydrate from tobacco-leaf fibre on incubation with 'trypsin'*

(3 g. lots of fibre (dry matter, 21.2%; N, 5.0% of dry matter) + 9 ml. 0.2M-sodium phosphate buffer (pH 7.5) + 2 ml. 2M-NaCl + 6 ml. 1% (w/v) trypsin solution + 3 ml. water. Trypsin replaced by water in the control. Four incubations of 24 hr. each, fibre then milled and extracted with 20 ml. water. Fibre reincubated with trypsin for 24 hr. Extracts after milling spun at 8000 rev./min. (6400 g) before doing estimations.)

	With trypsin mg./100 mg. dry fibre			Control mg./100 mg. dry fibre		
	Nitrogen	Carbohydrate	Reducing sugar (as glucose)	Nitrogen	Carbohydrate	Reducing sugar (as glucose)
First incubation	1.51	10.80	8.10	0.44	1.80	1.01
Second incubation	0.91	2.00	1.64	0.19	0.98	0.44
Third incubation	0.38	0.75	0.41	0.19	0.63	0.32
Fourth incubation	0.19	0.60	—	0.09	0.25	—
Mill extract	0.25	1.38	1.10	0.19	1.29	1.01
Fifth incubation	0.31	1.64	0.50	0.35	6.30	4.12
Residue	1.17	—	—	3.16	—	—

Table 3 gives the results of an experiment in which minced fibre was incubated four times with pancreatic amylase, then milled and extracted with water and finally reincubated with pancreatic enzyme. It appears from a comparison of the trypsin and control sections of Table 3 that the extract obtained by milling is substantially the same in each, and probably represents the release of reducing sugar from the interstices of the material. Further incubation with trypsin results in little further polysaccharase action, as most of the carbohydrate released is non-reducing. This carbohydrate is probably a fraction from the cell wall, as less carbohydrate is released from milled extracted fibre on incubation with purified polygalacturonase than from minced fibre.

LEAF PROTEIN

Fibre protein may be divided arbitrarily into three categories with differing properties: cytoplasmic protein contained in intact cells or secondarily precipitated on to insoluble material, chloroplast protein in the form of intact chloroplasts, and perhaps protein bound to the fibre itself (cell-wall protein). In addition, the fibre will contain non-protein nitrogenous substances, such as chlorophyll.

When crude sap is centrifuged a number of layers are visible in the deposit. The top layer is dark green and appears to consist almost entirely of broken-up cell contents rich in chlorophyll. Beneath this is a layer of starch granules, and at the bottom of the tube a small amount of dark material, probably dirt from the surface of the leaves.

The top dark-green layer, which will be referred to as

recentrifuged four times, the layer of larger particles at the bottom of the sediment being rejected. The material finally got was exhaustively extracted with acetone. A sample of the fibre was, after four washes with water, also extracted until no more green colouring matter could be removed. Portions of the acetone extracts were diluted with acetone until their colour appeared the same in a visual colorimeter, matching both the transmitted green colour and the transmitted red colour observed with greater depth of solution. Total N was determined on the white residues from extraction, and the pigment/N ratio calculated. If all the chlorophyll remaining in the fibre had associated with it as much N as in the sap sediment, 80% of the N of fibre would be accounted for. There is no reason to think that any chlorophyll exists in the leaf outside the chloroplasts; this suggests therefore that 80% of the N of fibre is due to chloroplasts that have not been released.

Cytoplasmic protein flocculates on standing. Extensive flocculation on to both fibre and chloroplast during the preparation of fractions would vitiate this hypothesis. The fact that finely divided chromoprotein remained suspended in the supernatant from the preparation of the chloroplast fraction was regarded as evidence that flocculation was not extensive, for flocculation invariably results in the removal of suspended chromoprotein when sap is allowed to age. Assuming that 80% of the fibre N does represent chloroplast material, the remaining two fractions—cytoplasmic protein and cell-wall protein—cannot exceed 20%. It is probable, therefore, that protein associated with the chloroplasts is quantitatively the most important in fibre.

Leaf protease

The protease of the tobacco leaf has been investigated before (Tracey, 1948), and its properties are such that little detectable activity can be expected under the conditions used in experiments described in this work either in sap fractions or in fibre.

Pancreatic protease (commercial trypsin)

Action on fibre. The same conditions were used in following protease action as for amylase action. Three incubations of minced fibre removed up to 80% of the fibre N and 40% of the dry matter, but the percentage removed was very variable, depending on the initial N and starch content. After exhaustive treatment with trypsin, fibre still contains 0.8–1% of the dry matter as N. The effect of prolonged incubation with crude trypsin followed by milling and re-incubation on N liberation is shown in Table 3. Little N is removed on extraction after milling and on re-incubation. The amount is of the same order as that released from the control fibre, and may be due to enzyme action or to gradual solution of protein.

Action on chloroplast fraction. Autolysis of the chloroplast fraction leads to the appearance of some N in solution, but prolonged incubation with trypsin does not render all the N soluble (Table 4). The residue from chloroplasts

extracted with 2N-HCl for 1 hr. at room temperature to remove calcium oxalate and other Ca salts, after incubation with trypsin contains about 4% N on dry matter, a small part of which is in the form of chlorophyll. The composition of the chloroplast fraction naturally varies with the previous illumination of the leaves. Since the chloroplast fraction contains a considerable proportion of starch, amylase action of the pancreatic enzyme is also prominent.

After prolonged incubation at pH 7.2 in phosphate buffer, carbohydrate becomes soluble. The addition of trypsin or saliva does not increase the amount of carbohydrate liberated, but does increase the rate of liberation (Table 5). After incubation of the chloroplast layer with trypsin, centrifuging results in the formation of three layers. The top is composed of free chloroplasts, the middle of calcium oxalate granules, and the bottom of masses of chloroplasts still apparently held together.

LEAF LIPIDS AND PANCREATIC LIPASE

The effect on tobacco-leaf fibre of the lipase present in crude trypsin solutions has not been studied in detail. Fibre was extracted with acid ethanol-ether (100 vol. ether:100 vol. 95% (v/v) aqueous ethanol:1 vol. 10N-HCl) until no more pigment appeared in solution. The solvent was evaporated and the residue extracted with CHCl_3 ; the dry matter thus dissolved was determined. The material

Table 4. *Fractionation of a sample of chloroplast layer from tobacco-leaf sap*

(About 250 mg. dry matter was incubated at pH 7.0 for three periods of 5 days at 35° with three successive lots of trypsin. Autolysis was in 0.2M-sodium phosphate buffer (pH 7.0) for the same time. No polyuronide was detected in the material. N as percentage of initial dry matter was 4.62%. 14.8% of this N remained in the residue before ether and acetone extraction after HCl-trypsin, 12% after trypsin and 67.5% after autolysis.)

	Treatment		
	Washed 2N-HCl (1 hr., room temp.) then trypsin	Trypsin	Autolysis at pH 7.0
Percentage of initial dry matter found in solution as carbohydrate (measured as glucose and multiplied by 0.9 to convert to polysaccharide)	41.3	38.7	21.1
N in solution $\times 6.25$ as percentage of initial dry matter	24	26	8
Wt. of residue not soluble in acetone and ether as percentage of initial dry matter	9.4	20.7	51
Wt. of residue soluble in acetone and ether as percentage of initial dry matter	7.8	7.6	10.5
(Ca^{++} + oxalate) found in HCl washings as percentage of initial dry matter	11.6	28.3	61.5
Totals of components	94.1	93.0	90.6

Table 5. *Incubation of chloroplast layer from tobacco-leaf sap with trypsin and saliva*

(Chloroplast layer (about 150 mg. dry matter) was incubated at 35° with 5 ml. 0.2M-sodium phosphate buffer (pH 7.2) + 5 ml. water or trypsin solution (1% w/v) or saliva. Total volume 10.6 ml. After each incubation the supernatant after centrifuging was removed and buffer and enzyme solutions replaced.)

	Carbohydrate liberated (mg./100 mg. initial dry matter) (figures in brackets show carbohydrate liberated in each incubation as a percentage of total carbohydrate liberated in all three incubations)		
	No enzyme	Saliva	Trypsin
First incubation (3 days)	12.0 (51)	21.5 (93.5)	19.4 (87.0)
Second incubation (2 days)	7.9 (33.5)	1.5 (6.5)	2.5 (11.0)
Third incubation (6 days)	3.7 (15.5)	0 (0)	0.4 (2.0)
Totals	23.6 (100)	23.0 (100)	22.3 (100)

soluble in CHCl_3 varies between 6 and 9% of the dry matter, whether fresh fibre or trypsin-treated fibre is used. Thus CHCl_3 -soluble material is lost at about the same rate as total dry matter during treatment with trypsin. The CHCl_3 -soluble material contains 1-2% of its dry matter as nitrogen.

DISCUSSION

Either salivary amylase or pancreatic amylase is satisfactory as an agent for the removal of starch from unboiled fibre. Though tobacco-leaf starch is more rapidly attacked after boiling, it is attacked in the intact state much more rapidly than is potato-tuber starch. This is possibly due to the small grain size, or because the storage starch of a tuber may have more resistant outer layers.

Softening of the fibre by trypsin is not readily measurable, but is obvious to the senses. Since salivary amylase does not have this effect, it must be due to enzymes other than amylase. The most likely are the proteases since it has been shown (Bawden & Pirie, 1946) that chymotrypsin has a similar effect.

Nitrogenous substances present in washed fibre may be divided for convenience into three classes: water-soluble substances trapped in the interstices of the fibre, water-insoluble substances associated with the chloroplasts and their aggregates which give the fibre its green appearance, and water-insoluble substances associated with the cell wall. The first of these groups may be supposed to be similar to the nitrogenous substances of the supernatant of centrifuged sap, about half of which are protein. The second group corresponds with sap sediment (after removal of cell-wall debris by differential centrifugation), which will contain protein nitrogen and chlorophyll nitrogen, and possibly other non-protein nitrogen. About the third group there is little evidence. Both cuticle and fibrous tissue freed from protoplasts and chloroplasts contain water-insoluble nitrogen, some of which may be protein nitrogen though there is no evidence that it is. The fibrous tissue may also contain nitrogenous

lignin of the type found by Bondi & Meyer (1948) in young plants. It is unlikely that much of the first (occluded sap) fraction will be present after thorough washing, or that it would have any effect on fibre texture. If protein is associated with the cell wall, then its removal by trypsin might account for the observed change in texture. The second fraction, however, is quantitatively important, perhaps accounting for three-quarters of the fibre nitrogen if it is assumed that the chlorophyll/insoluble nitrogen ratio of sap sediment can be used to give a measure of its amount in fibre. The fact that softening of the fibre occurs early in the course of trypsin digestion also suggests that it may be the partial digestion of the bulky, readily available second fraction that accounts for the softening of fibre by trypsin action.

About 80% of the nitrogen of crude fibre, and of the deposit of protoplasts from saps (analogous to the second, predominating fraction in crude fibre) is rendered soluble by trypsin, so that a distinction between the fractions is not possible in this respect. Figures given by White, Weil, Naghski, Della Monica & Willaman (1948) for the digestion by trypsin of protoplasts isolated from the leaves of a number of plants by fermentation with *Clostridium roseum* range from 27 to 81% of the protein nitrogen.

SUMMARY

1. All starch can be removed from tobacco-leaf fibre by incubation with salivary amylase.
2. All starch and about 80% of the total nitrogen can be removed from tobacco-leaf fibre by incubation with commercial trypsin.
3. Incubation of the fibre with commercial trypsin leads to a loss of chloroform-soluble material.
4. Figures are given for the composition of the particulate matter, chiefly chloroplasts, in crude tobacco-leaf saps.
5. The nature of the nitrogenous substances of the fibre is discussed.

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